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Work form this laboratory demonstrated that receptors with integrin epitopes, and that bind to the consensus RGD (arginine-glycine-aspartate) sequence, are concentrated in forebrain synapes. Light and electron microscopic immunocytochemical experiments from other groups then showed that subunits are present in hippocampus and that subunits are highly concentraed in hippocampal synapses. A critical issue for adhesion hypothesis of LTP consolidation concerns the route whereby extant adhesive relationship are disrupted so as to allow new configurations to emerge. This is less of problem for integrins that for NCAMs because breakdown of the spectrin network by calpin would have profound effect on relationships between the integrins and the sybsynaptic cytoskeleton. AFOSR supported work has now identified a route whereby the triggers for LTP could also relax NCAM mediated adhesion.

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March 21, 2000

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RE: Final Technical Report for F49620-95-1-0304

Dear Ms Veon,

Enclosed is the Final Technical Report for Grant F49620-95-1-0304, Prinicipal Investigator, Gary Lynch. If you have any questions, you may call me at (949) 824-2281. Thank you.

Sincerely,

Cindy Wilson
Data Administrator

03-27-00P03:03 RCVD

Final Technical Report

cc: Lynn Brown
Cheryl Herrera

Progress Report September 1998

A. THE ADHESION RECEPTOR HYPOTHESIS OF LTP CONSOLIDATION.

1. Integrins and Synaptic Plasticity

a. Regional heterogeneity in the expression of integrin subunits. Work from this laboratory demonstrated that receptors with integrin epitopes, and that bind to the consensus RGD (arginine-glycine-aspartate) sequence, are concentrated in forebrain synapses [e.g., Bahr et al., 1997]. Light and electron microscopic immunocytochemical experiments from other groups then showed that $ß_1$ subunits are present in hippocampus and that α_8 subunits are highly concentrated in hippocampal synapses. Having established that integrins are indeed located in adult synapses, it was appropriate to ask what subtypes are used by brain and to map their distributions across regions. The question is potentially of considerable importance because the type of 'outside-in' signalling performed by integrins is affected by their subunit composition. Regional variations in integrins could, therefore, translate into variations in structural plasticity and neurotropism.

Integrins are dimers with α and ß subunits; 14 of the former and 8 of the latter have been identified. Eleven α units and seven ßs occur with sufficient frequency to be plausible candidates for brain integrin subunits. Radiolabelled probes for the mRNAs encoding the target subunits have been obtained or constructed and mapping work with *in situ* hybridization is underway. It is apparent from early results that the brain is highly differentiated with regard to integrin expression. For instance, labelling is dense in basolateral division of the amygdala but almost undetectable in corticomedial and central divisions. The distribution of α_v in cortex is complementary to that for α_8; i.e., dense superficially and absent in the deep zones. Some patterns have been identified; e.g., all brainstem motor nuclei have dense concentrations of $ß_1$, a subunit that is present at low levels in most other brain systems (Pinkstaff et al, 1998).

b. Integrin antagonists reverse LTP. Prior work implicated the synaptic integrins in LTP consolidation by showing that diverse peptide antagonists of RGD binding prevent the formation of stable potentiation without affecting synaptic potentials or the complex physiological responses to theta bursts. The dose dependency of these effects corresponded to that for peptide suppression of integrin mediated adhesion in various tissues. Similar sized peptides with no relationship to the RGD site did not interact with LTP [Bahr et al., 1997]. Assuming from these observations that integrins contribute to consolidation, it can be expected that the peptide antagonists would (i) reverse long term potentiation in a time dependent fashion, and (ii) be effective over the same time period as are hypoxia and low frequency synaptic activity. This was tested by microperfusing an integrin antagonist to one of two recording sites in hippocampal slices at various times before and after long term potentiation (LTP) was simultaneously induced at both sites. Applications ten minutes before, immediately after, and ten minutes after induction caused LTP at the experimental site to decay steadily relative to that at the within-slice control site. However, microperfusion at twenty-five or more minutes after induction had no detectable effect on potentiation. Similar results were obtained when the integrin antagonist was perfused into the slice rather than topically applied. Comparable experiments using a peptide that blocks an extracellular binding site of neural cell adhesion molecules did not yield time dependent reversal of LTP; i.e., the antagonist blocked LTP when applied before induction but not afterwards (Staubli et al, 1998). These results constitute strong evidence that integrin activation is a critical step in the consolidation of LTP. The close correspondence between the time courses for LTP reversal by the antagonists versus low frequency stimulation also strongly suggests that the two effects are linked; i.e., that synaptic activity erases LTP by deactivating or blocking integrins.

2. The Triggers for LTP Cause the Extracellular Proteolysis of Neural Cell Adhesion Molecules (NCAMs). A critical issue for adhesion hypotheses of LTP consolidation concerns the route whereby extant adhesive relationship are disrupted so as to allow new configurations to emerge. This is less of a problem for integrins than for NCAMs because breakdown of the spectrin network by calpain would have a profound effect on relationships between the integrins and the subsynaptic cytoskeleton. AFOSR supported work has now identified a route whereby the triggers for LTP could also relax NCAM mediated adhesion. Specifically, a 30 sec application of NMDA to cultured hippocampal slices caused a rapid and pronounced increase in the concentration of a 75 kDa polypeptide recognized by polyclonal antibodies raised against NCAMs. The 75 kDa protein is smaller than intact NCAM isoforms (120-180 kDa) but larger than their cytoplasmic domains, indicating that the fragment incorporates a significant portion of the extracellular segment of the parent molecule. In agreement with this, an antibody raised against the fibronectin type III repeat region of NCAM labeled and immunoprecipitated the 75 kDa species. Also pointing to an extracellular origin, the cleavage product was present in soluble (high speed supernatant) fractions from the

slices. Samples collected 30 seconds after a 30 second infusion of NMDA contained approximately twice the concentrations of the 75 kDa fragment than samples from control tissue. Finally, formation of the fragment is blocked by a selective inhibitor of serine proteases (Hoffman et al, 1998).

Stimulation of extracellular proteolysis by NMDA receptors is surprising given that the receptors initiate biochemical changes by admitting calcium *into* the intracellular compartment. Nonetheless, all evidence points to initial cleavage occurring at the consensus serine protease site found extracellularly near the transmembrane segment of NCAMs. The most probable explanation is the secretion of a serine protease in response to NMDA receptor stimulation. In any event, extracellular cleavage would suspend the influence of the NCAMs on synaptic architecture and thus open the way to synaptic reorganization and stable synaptic potentiation. Future work will have to test the prediction that drugs which block the extracellular proteolysis of NCAMs prevent the stabilization of LTP.

3. Testing Hypotheses Regarding The AMPA Receptor Changes Responsible For Stable Expression Of LTP

Four hypotheses – membrane environment, modulatory proteins, receptor clustering, receptor insertion/ activation – have been formulated with regard to how the consolidated state of the synapse amplifies the response of AMPA receptors to released transmitter. While underspecified, the first two ideas are, in principle at least, testable. Techniques are now available for applying agonists for about the duration of a release event during synaptic transmission (≈ one millisecond) to a membrane patch containing 500-1000 AMPA receptors. The resultant evoked currents closely resemble excitatory post synaptic currents. Moreover, experiments conducted during the tenure of the AFOSR award show that manipulations which alter the size or waveform of the patch responses to millisecond transmitter pulses produce very similar effects on the size and waveform of synaptic responses [Arai and Lynch, 1996, 1998a, Arai et al., 1996a]. Accordingly, synaptic elements that modify AMPA receptors so as to produce potentiated responses *in situ* should produce qualitatively similar 'potentiation' of patch responses to very brief applications of glutamate.

The significance of producing 'potentiation' in an excised patch is directly related to the stringency of the definition of long term potentiation. There are a number of treatments that alter AMPA receptor binding and it can be assumed that at least some of these will affect the current flow. Criteria in addition to enhanced responses are needed to establish that a given manipulation creates an LTP-like effect. It is pertinent in this regard that LTP, in addition to increasing amplitudes, also changes the waveform of the synaptic response and the manner in which it is altered by AMPA receptor modulators (Kolta et al, 1998; also see past AFOSR reports). These observations impose a demanding set of criteria that must be satisfied before a treatment can be said to have reproduced LTP.

B. USES FOR AMPA RECEPTOR MODULATORS

1. Applications For Acute Treatments With Ampakines.

Work supported by the AFOSR grant has shown that ampakines shift the balance of activity between cortex and striatum to favor the cortex [Palmer et al., 1997]. Enhancing or restoring 'cerebral dominance' could have positive effects in regulating subcortical regions responsible for motoric, emotional, and autonomic functions. If so, then the ampakines would constitute a pharmacology developed for LTP but having applications beyond learning and memory. The idea that using ampakines to facilitate cortical circuitries engaged by particular tasks or environmental demands will have a positive outcome on behavior was subjected to four tests during the tenure of the AFOSR grant.

- Experiment One showed that the excess arousal and perturbations of the striatal motor functions induced by methamphetamine are substantially reduced by ampakines [Larson et al., 1996]. This result, which has been replicated and extended by other groups, has prompted clinical trials testing ampakines as a treatment for schizophrenia.
- Experiment Two measured the speed with which overtrained middle aged rats collected rewards in an eight arm radial maze. Scores were reliably improved on drug days, primarily because the rats spent less time in extraneous behaviors [Davis et al., 1997].
- Experiment Three asked if ampakines can be used to offset declines exhibited by middle aged rats in the performance of a complex behavior involving a prominent social component; i.e., copulation. Positive results were obtained in seven of eight rats (Granger et al, in prep).
- Experiment Four assessed the behavior of rats placed in a novel environment in the middle of the sleep portion of their day-night cycle. Ampakines caused a statistically significant increase in exploratory behavior in this pilot study (Granger et al, unpublished data).

The above results raise the possibility that ampakines will have positive behavioral effects whenever activity in the exceedingly complex networks running through the cortical telencephalon is suboptimal for the circumstances now present.

2. Correlating the Biophysical and Behavioral Effects of Positive Modulators.

Work carried under the AFOSR grant established that modulators which slow the deactivation of AMPA receptors produce an equivalent prolongation of synaptic responses elicited by single afferent discharges and that compounds which act on desensitization (cyclothiazide; potassium thiocyanate) have little effect on single responses. Still more recent work found that drugs which accelerate resensitization do facilitate the later responses to high frequency bursts of afferent stimulation (Arai and Lynch, 1998b). Since bursts of these kinds are used to induce LTP, it follows that positive modulators with predominant effects on desensitization/resensitization should promote the induction of potentiation while having little effect on network activities not involving high frequency bursting. Such compounds could be significantly more selective in enhancing memory than are those ampakines that slow deactivation and facilitate synaptic responses to single afferent discharges.

3. Correlating Receptor Subunit Preferences of Positive Modulators with their Influence on Behavior.

Clonal cell lines expressing AMPA receptors composed of five copies of a single subunit (homomeric pentamers) have been constructed for the purpose of testing for subunit preferences (e.g., GluR1 vs GluR2 vs GluR3) amongst the now more than 100 physiologically active ampakines (see past reports). Modest but real subunit preferences in the ampakine family were identified in the first studies using the panel [Hennegriff et al., 1997]. Recent work has identified more clearcut preferences and differences between ampakines in this regard (Arai et al, in prep). Receptor autoradiography has been used to confirm that preferences for splice variants are reflected in ampakine preferences within hippocampus and cortex. That is, drugs that differentiate between 'flop' and 'flip' variants of the receptors have a greater effect on ligand binding in those regions in which their preferred variant is concentrated (Kessler et al., 1998). The panel of cell lines has recently been extended to include each of the eight major subunits known for forebrain; i.e., GluR 1,2,3,4, with 'flip' and 'flop' splice variants for each case.

4. A New Family of Positive Modulators

An entirely new family of positive modulators ("D-drugs") with little structural similarity to the ampakines has been designed and synthesized during the tenure of the current AFOSR grant. The motivation for this relates to the points made in the above three sections: The greater the range of subunit preferences and biophysical selectivities, the greater will be the likelihood of developing drugs targeted for specific brain regions and functions. The D-drugs began with an analysis of the structure of various benzothiadiazides followed by the application of combinatorial chemistry. The new drugs are in an early stage of development but variants sufficiently potent to be used in behavioral work are already available. Pilot studies indicate that the compounds reproduce the minimal ampakine effects: changes in exploratory activity with seizures at much higher dosages. Side effects not found with the ampakines have been seen and efforts are being made to synthesis away from these and to achieve greater potencies. The D-drugs provide an entirely new set of tools with which to explore how effects on receptor classes and receptor kinetics relate to effects on behavior and brain neurochemistry (Phillips et al., in prep, Arai et al., in prep.).

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